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Review

Optomechanical devices for deep plasma cancer proteomics

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ABSTRACT

Most of the cancer deaths could be avoided by early detection of the tumor when it is confined to its primary site and it has not metastasized. To this aim, one of the most promising strategies is the discovery and detection of protein biomarkers shed by the young tumor to the bloodstream. Proteomic technologies, mainly mass spectrometry and multiplexed immunoassays, have rapidly developed during last years with improved limits of detection and multiplexing capability. Unfortunately, these developments together major investments and large international efforts have not resulted into new useful protein biomarkers. Here, we analyze the potential and limitations of current proteomic technologies for detecting protein biomarkers released into circulation by the tumor. We find that these technologies can hardly probe the deepest region of the plasma proteome, at concentrations below the pg/mL level, where protein biomarkers for early cancer detection may exist. This clearly indicates the need of incorporating novel ultrasensitive techniques to the proteomic tool-box that can cover the inaccessible regions of the plasma proteome. We here propose biological detectors based on nanomechanical systems for discovery and detection of cancer protein biomarkers in plasma. We review the modes of operation of these devices, putting our focus on recent developments on nanomechanical sandwich immunoassays and nanomechanical spectrometry. The first technique enables reproducible immunodetection of proteins at concentrations well below the pg/mL level, with a limit of detection on the verge of 10 ag/mL. This technology can potentially detect low abundance tumor-associated proteins in plasma at the very early stages of the tumor. The second technique enables the identification of individual intact proteins by two physical coordinates, the mass and stiffness, instead of the mass-to-charge ratio of the protein constituents. This technology enormously simplifies the identification of proteins and it can provide useful information on interactions and posttranslational modifications, that otherwise is lost in mass spectrometry.

1. Introduction

Cancer is a leading cause of death globally. The World Health Organization (WHO) estimates that approximately nine million people die of cancer every year [1,2]. Most of these lives could be saved if patients had timely access to early detection. The tumor at early stage is localized at the organ of origin and can be easily resected. In addition, treatments are more efficacious. Unfortunately, tumor at early stages use to be asymptomatic. At later stages the tumor invades surrounding tissue and metastasizes to distant organs. When metastasis occurs, it creates complications that account for the vast majority of deaths from cancer [3]. Without early detection, treatment costs rise substantially, resources are used inefficiently and the need for palliative care services increases.

Cancer initiation and progression is regarded as a multi-step process, which is reflected by progressive genetic alterations that drive the transformation of normal human cells into highly malignant derivates [4]. Mutations, amplifications or deletions in these genes may lead to a de-coupling of biological mechanisms involved in the regulation of normal cell growth and differentiation. These changes are reflected into alterations in the gene and protein expression patterns of the tumor cells. Identification of the genetic and proteomic modifications associated to cancer, referred to as cancer biomarkers, is crucial for detection of the disease, choice of the optimal therapy and more precise prognostication of disease progression [5,6]. Nucleic acid biomarkers include gene mutations, gene methylation, gene overexpression and chromosomal aberrations. Despite the increasingly refined technologies for discovery of nucleic acid biomarkers, biomarkers for early cancer detection still have not been found. A fundamental reason for this disparate progress is that the gene expression is not always correlated with the actual abundance of proteins, the functional components within the organism. Compared to nucleic acids, proteins reflect more precisely the physiological state of cells and tissues. Therefore, research on cancer biomarker discovery is increasingly turning its attention on

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the discovery of protein biomarkers [7,8]. Another fundamental reason for this change of target is that a significant part of the proteins of every cell of the body is released to the blood stream through several mechanisms. In addition, blood is the sample of choice for medical diagnosis because of its easy accessibility and the complete laboratory infrastructure already developed for its analysis.

Discovery of protein biomarkers shed by the tumor at early stages in plasma could be the key for early cancer detection. Unfortunately, we are still far from achieving this chimera. Proteomic technologies, mainly mass spectrometry and multiplexed immunoassays, have developed rapidly during recent years with improved limits of detection and multiplexing capability. Unfortunately, these developments together major investments and large international efforts have not resulted into new useful protein biomarkers. A fundamental reason for this dismal progress is the extraordinary complexity of the human plasma that comprises more than 10 000 protein species with known concentrations ranging more than 10 orders of magnitude [9,10]. It is expected that biomarkers for early cancer detection are well-below the bottom of the current detection limits in proteomics. Although, the analytical capability of proteomic technologies is rapidly improving, we foresee the need of new ultrasensitive technologies that can access to the deepest part of the human plasma proteome.

Nanotechnology has provided in the last decade a wide variety of nanobiosensors that have shown ultrahigh sensitivity with small volumes of sample. The unprecedented sensitivity of these biosensors is capitalized by the reduction of the size of the sensing structures, and by the appearance of new phenomena at the nanoscale. Nanobiosensors raised large interest in the biomedical community for their potential to surpass the limitations of contemporary technologies to quantitate biomarkers at concentrations well-below the pg/mL level [11]. However, the promises of most of these technologies have not been translated into valid clinical tests. Many nanobiosensors have shown multiple pitfalls and issues regarding specificity, reproducibility and reliability. The time has arrived that research on nanosensors faces the real problems that arise in the long and uncertain path from the proof of concept up to achieving an assay of clinical utility. Detecting ultralow concentrations of biomolecules in complex mixtures poses a formidable challenge that requires of solving manifold problems including nonspecific adsorption, biological noise and the diffusion limit [12–14].

We here review recent advances in biological detectors based on nanomechanical systems that can revolutionize the discovery and detection of low-abundance protein cancer biomarkers in plasma. Nanomechanical systems are suspended micro- or nanoscale structures in which their position and motion is sensitively altered by minuscule forces such as the weak interactions between a pair of biomolecules [15,16]. Biological detectors based on nanomechanical systems exploit this exquisite sensitivity to translate biological signals into the mechanical domain [17–22]. Although these technologies have still not been used in clinical proteomics, we envision that their unique and unprecedented attributes make them promising candidates for tackling early cancer detection the next years. In order to see how nanomechanical systems can complement current efforts in proteomics, we will start the review by describing the existing knowledge about the human plasma proteome and protein biomarkers as well as the employed technologies to discover protein biomarkers in plasma. Then we will review recent advances on nanomechanical biological detectors that can contribute to the discovery of new protein biomarkers in plasma.

2. Human plasma proteomics

Human proteomics aims to identify and quantify all the proteins expressed by ~20,300 human protein-coding genes predicted from the human genome and their corresponding protein isoforms [23]. In comparison to genomics, proteomics has advanced at much slower rate. Genomics rapidly progressed due to its powerful biotechnological arsenal that includes synthesis of nucleic-acid probes, methods for amplification of few copies in complex mixtures and the development of constantly refined technologies for nucleic acid sequencing. Unlike DNA, which is subject to one major form of modification (methylation), proteins are more complex structures that undergo various types of post-translational modifications such phosphorylation, acetylation and glycosylation— each of which is capable of producing a functional shift that potentially affects disease development, progression and therapeutic response. Moreover, no methods exist for protein synthesis, amplification nor sequencing. Only until very recently, proteomic technologies based on mass spectrometry and antibody arrays have been able to provide reliable information about the human proteome. In 2014, first drafts of human proteome were described based on analysis of mass spectrometry data [24,25]. Later, in depth analysis of these publicly available drafts has shown significant pitfalls that led to an overestimation of the identified protein-coding genes [26]. The formidable complexity of deciphering the human proteome led to the launch of large-scale international initiatives such as the human proteome project (HPP) by the Human Proteome Organization's (HUPO) [27]. In 2015, one of the primary HPP information resources, the Human Proteome Atlas programme, presented the first map of the human tissue proteome [28]. About 38% of the genes encode both secreted and membrane-bound proteins. This fraction of the proteome is particularly interesting as aberrant secretion or shedding of membrane-bound proteins with an extracellular domain is a fundamental mechanism by which these proteins can be elevated in blood at early stages of a tumor [5]. A classic example is the cell membrane protein HER2, whose overexpression is associated with high risks of relapse and death from breast and ovarian cancers. HER2 is the target of therapeutic monoclonal antibody trastuzumab. In 2000, the US Food and Drug Administration (FDA) approved a blood test for measuring circulating levels of HER2 for monitoring patients with metastatic breast cancer. Currently, it is estimated than roughly 15% of the predicted proteins by the human genome are still unidentified [29]. These missing proteins can be also a valuable source of future cancer biomarkers.

Plasma proteome represents the most comprehensive subproteome for discovery of biomarkers for early cancer detection [9,10]. It harbors proteins secreted or released from almost all tissues, along with proteins derived from infectious organisms and parasites residing inside the body. Every cell in the body leaves a record of its physiological state in the products it sheds to the blood [7]. Thus, the goal of early cancer detection is to find in blood tumor products that can be used as unambiguous signatures of the development of a tumor in some part of the body. The comprehensiveness of the plasma proteome, together its easy accessibility and simple collection, makes plasma the body fluid of choice for future screening tests for cancer diagnosis. Unfortunately, our existing knowledge about the human plasma proteome remains very incomplete. Currently, we know that approximately 99% of the plasma comprises 22 highly-abundant proteins including albumin (that makes up 55%), immunoglobulins, transferrin and haptoglobin [9,10]. The concentration of the highly-abundant proteins ranges from ~10 μg/mL to ~10 mg/mL. The remaining 1% is estimated to contain more than 10,000 proteins, including tissue-derived proteins (e.g. clinically used cancer biomarkers) in the ng/mL range and cytokines and interleukins in the low pg/mL range. The wide dynamic range of plasma proteins, at least 10 orders or magnitude, as well as the heterogeneity and complexity of plasma, makes plasma the most difficult sub-proteome to characterize.

The Plasma Proteome Database (PPD, http://www.plasmaproteomedatabase.org/) was developed as a part of Human Proteome Organization's (HUPO) initial effort to characterize human plasma proteome [10]. The PPD contains data of the plasma protein concentration for 1278 proteins (Fig. 1). We observe that the number of identified proteins increases as the concentration approaches to 1 ng/mL, and then abruptly declines for lower concentrations. This change of

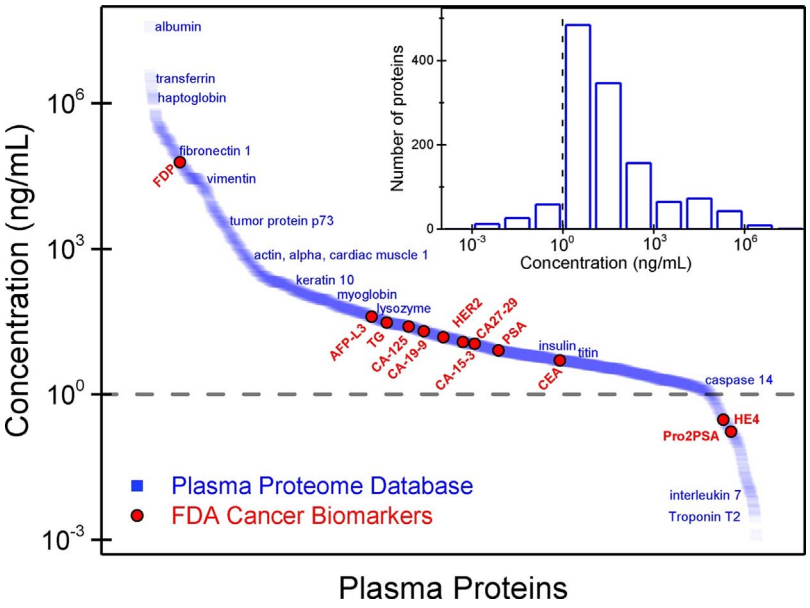

Fig. 1. Plasma protein concentration for the 1287 proteins reported in the PPD (http://www.plasmaproteomedatabase.org/). The inset shows the number of proteins reported per log of concentration. The dashed line approximately represents the lowest concentration at which our knowledge on the plasma proteome becomes to decline. Red symbols indicate FDA-approved protein tumor markers for blood tests, which are described in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

trend in the number of discovered proteins is a clear manifestation of the technological limitations for characterizing the plasma proteome at concentrations well-below the ng/mL level.

Proteomic technologies have rapidly developed during recent years with improved limits of detection and multiplexing capability. These technologies can be split into two categories: mass-spectrometry-based technologies and multiplexed immunoassays. Fig. 2 shows the contribution of these technologies to our existing knowledge on the protein concentration in plasma (PPD). This graph reflects the state-of-the-art in plasma proteomics. Clearly, mass-spectrometry techniques dominate the discovery of plasma proteins, representing the 88% of the PPD entries. However, the number of proteins detected by mass-spectrometry quickly falls for concentrations below 1 ng/mL, whereas immunoassay methods enable to quantitate proteins in plasma up to near the pg/mL level. We below analyze the power and limitations of these technologies.

2.1. Mass spectrometry-based technologies

The generic overall process for identification and quantification of proteins using mass spectrometry (MS) [30–36] is sketched in Fig. 3(a). Proteins are first digested to peptides by sequence-specific enzymes such as trypsin. The resulting peptide mixture is separated by high-performance liquid chromatography (HPLC) that is on-line coupled to an electrospray- ionization (ESI) needle to convert the peptides into gas-phase peptide ions that are introduced into the vacuum of the mass spectrometer. However, most highly resolving HPLCs are unable to separate the tens to hundreds of thousands of different peptide species generated by the digestion, and thus many peptides will co-elute from the column and co-ionize simultaneously. This effect largely affects the performance characteristics of the MS data-acquisition strategies [35]. The mass-to-charge (m/z) ratios of the peptides are obtained at high resolution by ion trap mass analyzers such as Orbitrap hybrids or quadrupole time-of-flight (TOF). Peptide identification requires its fragmentation by dissociation methods such as collision-induced dissociation or electron-transfer dissociation. Peptide identity is derived by computational methods from the combined information of the m/z spectra of the precursor peptide (MS spectra) and of the corresponding fragment ions (MS/MS spectra). The computational methods compare the data with predictions calculated for each possible linear peptide

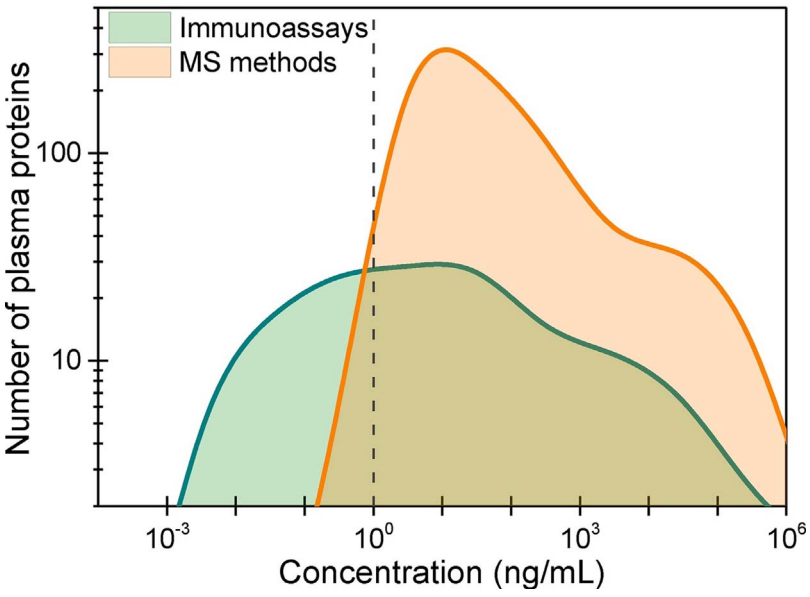

Fig. 2. Contribution of the two main classes of proteomic technologies to the quantification of proteins in plasma. Data source: PPD (http://www.plasmaproteomedatabase.org/).

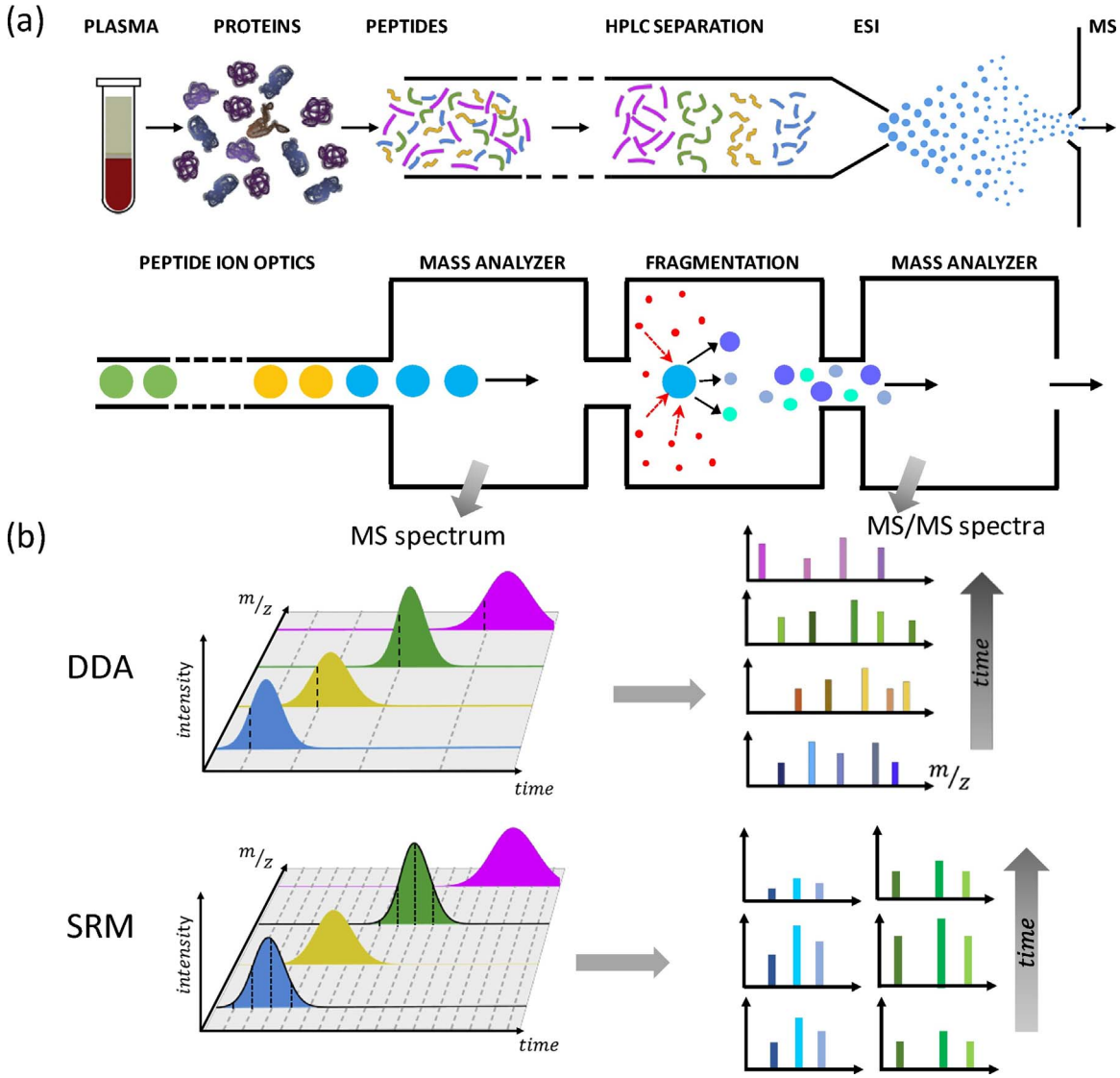

Fig. 3. Schematics of MS-based proteomics and data-acquisition strategies. (a) Outline of the generic workflow in MS-based plasma proteomics. At the top, the sample preparation process is shown. Plasma proteins are digested and the resulting mixture of peptides are separated by HPLC and introduced in the mass spectrometer by ESI. At the bottom, the sequential process events undergone by each peptide ion in the mass spectrometer are shown. The peptide ions are guided by ion optics to the first mass analyzer that provides the mass-to-charge ratio (m/z), then they are fragmented and the fragments are measured in a second mass analyzer. The data obtained in the first and second mass analyzers are referred to as MS spectrum and MS/MS spectra, respectively. (b) Structure of the data in the MS and MS/MS spectra in: the data-dependent acquisition mode used for shotgun proteomics (top) and the selected-reaction monitoring acquisition mode for quantitative targeted proteomics (bottom).

sequence and provide the most likely peptide identity.

Two general types of MS-based proteomics approaches are widely used for biomarker-related applications: global quantitative proteomics for biomarker discovery and targeted quantitative proteomics for candidate biomarker verification. We briefly review the two most important acquisition methods used in these approaches. We refer the reader to the many excellent review papers about MS-based proteomics for a more comprehensive survey of other signal acquisition methods that are gaining importance [31,33–36].

Global proteomics or shotgun proteomics uses data-dependent acquisition (DDA) that provides an unbiased and complete coverage of the proteome (Fig. 3b). In DDA, MS spectra of the precursor peptides are obtained, which result in a three dimensional map of the intensity versus m/z and HPLC separation time. The instrument operates in iterative acquisition cycles of about 1 s of the MS spectra and MS/MS spectra [34,35]. MS/MS spectra is only performed for each precursor for a single time point after precursor signal is above a certain threshold. The relative protein abundances can be determined by comparing signal intensity or peak area of corresponding peptides. However, the capability for quantification is poor because the ionization efficiency is dependent of the peptide sequence. In addition, the ionization efficiency of the peptides is modified when peptides enter ion source at the same time in a process called ion suppression. Quantification can be improved by sample labelling with different stable isotopes or by spiking isotopically label forms of the peptides of interest. The strength of the DDA method is the capability for routine detection of thousands of proteins, being the method of choice for uncovering complex proteomes and discovery of candidate protein biomarkers. However, DDA-based MS has inherent low reproducibility. For instance, automated precursor selection at a given chromatographic retention time introduces certain randomness due to variability in the chromatographic separation. Another major limitation is the undersampling due to the limited MS sampling duty cycle, which provides missing data, especially of the low abundance proteins.

Among the MS methods for targeted quantitative proteomics, we here highlight the selected reaction monitoring (SRM) [37,38] that was named method of the year 2012 by the journal *Nature Methods* [39]. In the SRM method, no complete MS/MS spectra are acquired (Fig. 3b).

Rather, a set of discrete predetermined fragment ion signals is repeatedly recorded for each predefined peptide over time [35,40]. The combinations of precursor *m/z* and fragment ion *m/z* pairs (typically three to five per peptide) are termed transitions. The lists of transitions are predefined in the acquisition method. Therefore, prior knowledge about the identity of the targeted peptides and of their fragmentation characteristics is required. The technique is best applied for consistently quantifying or validating the presence of targeted proteins, rather than discovering new peptides/proteins. SRM is typically performed on a triple quadrupole (QQQ) MS instrument where Q1 and Q3 serve as precursor ion and fragment ion filters, respectively, and Q2 acts as collision cell [34,36]. SRM is designed to achieve precise and accurate quantification (i.e., actual protein concentration) of the proteins by comparing signals with those of reference isotope-labeled synthetic peptides spiked in known amounts in the sample.

Mass spectrometry analysis of blood-derived plasma or serum poses a significant challenge in terms of sensitivity and analytical depth. Shotgun mass spectrometry has been the tool of choice for plasma proteomics due to its capability for identification of a large set of proteins with no prior knowledge required. However, quantification accuracy and reproducibility remain as limiting factors. Precursor selection in shotgun mass spectrometry analysis is biased towards the more abundant proteins in the plasma (concentrations above 1 μg/mL). Reducing the complexity of protein by depletion of the most abundant proteins enables identification of proteins at concentrations of 1–100 ng/mL. The counterbalancing problem is the inadvertent removal of other proteins, which could be due to peptides and proteins bound to the high-abundance proteins, especially albumin; cross-reactivity with the antibodies used for depletion of the high-abundance proteins; or non-specific binding to the used column or resin or dye [41]. Targeted mass spectrometry based on SRM is exclusively hypothesis driven, and thus it requires of the previous identification of the plasma proteome components by shotgun proteomics. It is focused on the detection and quantification of protein biomarkers previously detected in shotgun proteomics. In contrast with shotgun proteomics, MS based on SRM yields precise measurements of the protein concentrations with very low coefficients of variation and high reproducibility [42]. When applied to the plasma without any further depletion/enrichment step, SRM can achieve limits of detection of 0.1–1 μg/mL [36]. By applying depletion/enrichment steps, the limit of detection is at the ng/mL level. The fact that relatively few predetermined peptides (~10) are targeted per analysis constitutes the biggest strength (consistent quantification) but also the biggest limitation of the method.

Advances in instrumentation are providing mass spectrometers with higher scan speed and dynamic range. This enables new modes in data acquisition workflows such as the data-independent acquisition (DIA), which aims to acquire MS/MS spectra of all precursor peptides in the sample that are above the detection limit of the instrument [35,36]. This strategy promises to overcome the undersampling limitations of DDA used for shotgun proteomics and the limited number of peptides analyzed by SRM. This huge amount of data can be achieved by opening the precursor isolation window that breaks the “one MS/MS spectrum = one peptide” dogma of DDA and SRM. The technology offers a quantification accuracy similar to SRM with slightly worse sensitivity. The complexity of the data makes still challenging the high-throughput quantification of proteins in plasma [43]. Foreseeable advances in instrumentation and bioinformatics will have high impact in this acquisition mode.

2.2. Antibody-based plasma proteomics

Antibody-based proteomics [44–47] harness the extraordinary capability of the immune system to produce antibodies that recognize a unique molecule, referred to as antigen, of pathogens. Antibody-antigen recognition is based on spatial complementarity (lock and key) of the variable antigen-binding region of the antibody and a particular region of the antigen (epitope). The forces involved in this lock-and-key mechanism are weak interactions including electrostatic forces, hydrogen bonds, hydrophobic interactions and van der Waals forces. It has been estimated that humans generate about 10^{10} different antibodies (Abs), each capable of binding a distinct epitope of an antigen. Since the human proteome consists of approximately 20,500 non-redundant proteins, is feasible to foresee that multiplexed immunoassays is a powerful strategy to explore the proteome.

Antibodies can be produced by several methods, each having its advantages and disadvantages [47,48]. Polyclonal antibodies (pAbs) are generated by injection of the antigen/adjuvant conjugate to an animal of choice to initiate an amplified immune response. pAbs are heterogeneous, which allows them to bind to a wide range of epitopes of the target protein. However, the specificity of these antibodies is in many cases very low and it varies from batch to batch. Alternatively, monoclonal antibodies (mAbs) are made by identical immune cells that are all clones of a unique parent cell (e.g. cell culture that involves fusing myeloma cells with mouse spleen cells immunized with the desired antigen). Monoclonal antibodies are specific to a single epitope of the target protein. Another option is recombinant antibodies (rAbs) based on phage display technology. This technology involves recovering antibody genes from source cells, amplifying and cloning the genes into an appropriate phage vector, introducing the vector into a host (bacteria, yeast, or mammalian cell lines), and achieving expression of adequate amounts of functional antibody. Unlike polyclonal and monoclonal antibodies (mAbs), rAbs do not need hybridomas and animals in the production process. rAbs display many beneficial features for generation of antibody arrays, such as representing a renewable antibody source, the antibody library can be designed on a molecular level to display desired features and, very importantly, rAbs are very reproducible [48,49]. In fact, reproducibility of the antibodies has become a crucial problem as it is estimated that only a small percentage of commercial antibodies actually show required performance in terms of specificity [48,50]. Several online catalogues have been created in order to provide a valuable resource of antibodies. For instance, antibodypedia is a searchable database of more than 1,800,000 antibodies covering 94% of the protein-encoding genes that provides information on the effectiveness of specific antibodies in specific applications [50].

Enzyme-linked immunosorbent assay (ELISA) remains the gold standard for measuring protein concentration in human plasma [46,47,50,51]. ELISAs can be performed with a number of modifications to the basic procedure. The key step, immobilization of the antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody (cAb) that has been attached to the plate. The antigen is then detected either directly by an enzyme-tagged detection antibody (dAb) or indirectly by an enzyme-tagged secondary antibody that recognizes the detection antibody. The ELISA sandwich assay format provides higher specificity and sensitivity. In the final step, the enzyme substrate is added to produce a change of color of the surface that is used as the detection signal. Depending of the antigen, the limit of detection of ELISA typically ranges from 0.001 to 0.1 ng/mL and the dynamic range is from 2 to 3 logs. Based on ELISA, multiplexed immunoassays have been developed to quantify multiple antigens in a single sample of human plasma. These methods offer the advantage over MS-based proteomics of high sensitivity without fractionation or depletion of high-abundance proteins.

Multiplexed immunoassays for serum proteomics can be classified based on the solid support used for antibody immobilization: planar microarrays, in which cAbs are discretely immobilized on planar substrates (e.g. glass slides or microtitre plates) and antibody suspension bead arrays (SBAs) in which the cAbs are discretely immobilized in fluorescently coded microbeads [47,50–52]. In both formats, readout of the immunoassays is typically performed by measuring the fluorescence intensity of the immunoreaction tags. Fluorescent labelling formats can be split into direct and indirect labelling methods. In the first method, the sample proteins are tagged with fluorescent labels, thus avoiding

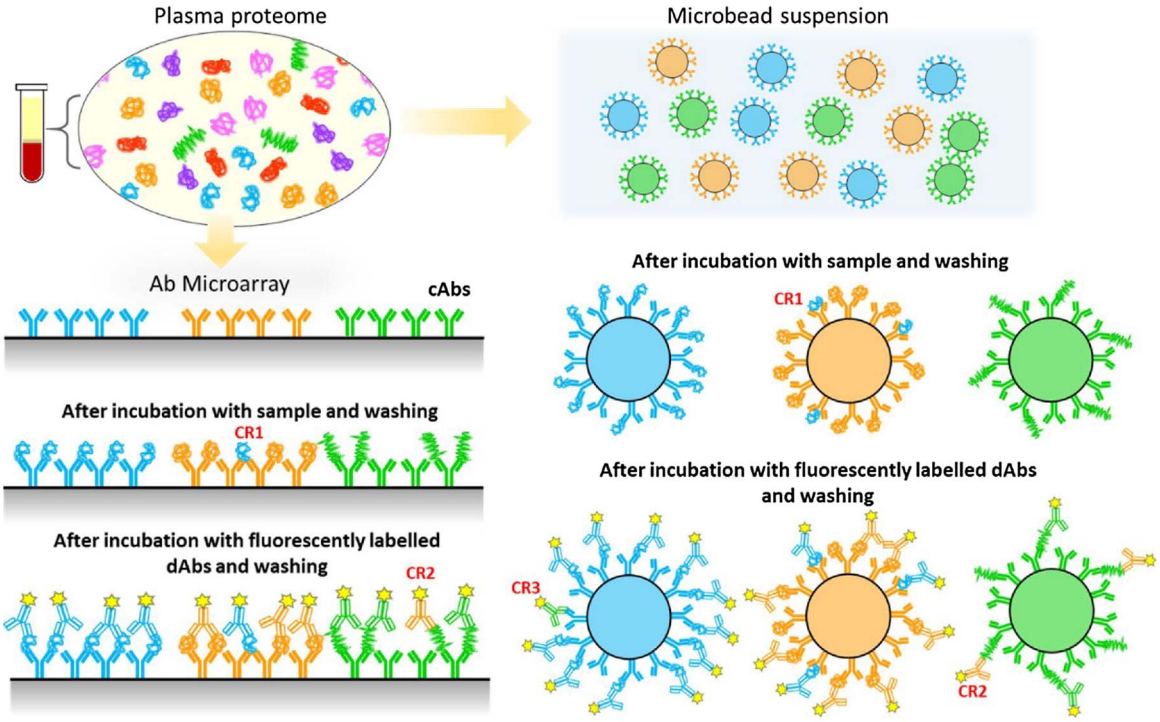

Fig. 4. Schematics of the indirect labelling method in Ab planar microarrays and Ab suspension bead arrays. The starting point for both multiplexed immunoassay formats is the plasma sample extracted from blood. Ab planar microarrays can be obtained by robotically printing cAbs upon an activated surface slide. SBAs employ plastic microbeads infused with a single (or several) chemiluminescent/fluorescent dyes and a functionally activated surface, prior to linking with a specific cAb. Beads functionalized with the same cAb have a unique fluorescent signature for subsequent identification by flow cytometry. Both multiplexed formats comprise similar steps to ELISAs: incubation with the sample, washing, incubation with the dAbs, washing and readout of the signal. Unlike ELISA, the dAbs are tagged with fluorescent molecules and readout is performed by fluorescent detection methods. SBAs feature the advantage that all the immunoassay steps are carried out in a flow chamber which enhances the immunoreaction kinetics and avoids Ab denaturation due to drying as it may occurs in planar microarrays. Both multiplexed immunoassay formats suffer from the cross-reactivity problem. Possible cross-reactions are labeled as CR. CR1 = Cross-reaction of a cAb with a noncognate protein. CR2 = Cross-reaction of a dAb with a noncognate protein. CR3 = Cross-reaction between a dAb and a cAb.

the need of dAbs that recognize a free region of the immunocaptured protein [52]. The direct labelling method allows detection of hundreds of proteins with a limit of detection of ~ng/ml. However, this technology is prone to nonspecific adsorption that gives rise to false positives and low reproducibility. The indirect labelling method is similar to ELISA sandwich assay, using fluorescently tagged dAbs for specific binding to the antigen recognized by the capture antibodies. The use of two specific antibodies per protein largely enhances the reproducibility, specificity and sensitivity. However, this method requires of the optimal identification of compatible antibody pairs with minimal cross-reactivity. Cross reactivity arises from the nonspecific binding between capture antibodies, detection antibodies and proteins [53]. It is estimated that the probability of cross-reactions scales up with the square of targets. In general, cross-reactivity contributes to an increase of the background noise, degrading the limit of detection and, in the worst cases, generating false positive signals. This limits the multiplexing capability to tens of proteins. A schematic of the indirect labelling method and cross-reactivities in antibody microarrays and SBAs is shown in Fig. 4.

Immobilization of capture antibodies in planar microarrays is performed by continuous flow microspotter systems and other advanced array printing technologies. However, the optimal recognition capability of these antibody microspots is compromised by denaturation of the antibodies due to drying of the microdroplets, inhomogeneous surface density and uncontrolled conformation of the antibodies. These effects, if uncontrolled, can lead to significant inter-assay variability. Nonetheless, antibody microarrays are suitable for large production and automated readout, which enables high-throughput analysis. In SBAs, the sample is mixed with plastic microbeads labeled with different fluorescent IDs and functionalized with capture antibodies. After all the incubation and washing steps, the microbeads are individually analyzed by a flow cytometry system, which features a dual-laser system to detect bead/captured protein ID. The bead array setup surmounts some of the limitation of planar arrays, such as printing artefacts and mass-transport limitations, which are abrogated by the conduction of the immunoassay in a liquid phase whereby the proteins are not denatured and the immunoreaction kinetics is improved, as agitation is readily executed. Currently, SBA constitutes the prevailing technology for FDA-approved multiplex protein analysis in clinical settings.

In addition to planar microarrays and bead assays, several strategies have been developed for addressing the cross-reactivity limit for multiplexing such as the use of aptamers instead of antibodies [54], proximity ligation assays in which the antibodies are tagged with DNA barcodes [55] or spatially localizing the immunoreaction steps [56], to name a few. These strategies reduce the cross-reactivity at the expense of a more complex and lengthy process.

2.3. *Where the biomarkers for early cancer detection are?*

Despite the large international efforts and major investments made over the last two decades for discovery of cancer protein biomarkers, only few biomarkers have been approved by the US Food and Drug Administration (FDA) for blood tests [57–61]. These biomarkers, represented as red dots in Fig. 1 are described in Table 1. Most of this handful of FDA approved biomarkers are not useful for early cancer detection [5,41,60]. Nonetheless, they are useful for monitoring disease evolution, response to therapy and recurrence of disease [6]. The reasons for this dismal progress are manifold including low reproducibility of the proteomics technologies, lack of quality standardization of the samples and misinterpretation by the bioinformatics tools of the big data generated by proteomics. These factors have made that most of recently proposed biomarkers fail when they are validated for clinical

Table 1
List of FDA-approved protein tumor makers used in clinical blood tests. The term "monitoring" stands for monitoring: disease status, disease progression, response to therapy. *Estimation of the concentration as the concentration is indirectly measured in ELISA in enzymatic units per volume unit. **Cut-off concentration depends on the concentration of fPSA and total PSA.

Biomarker	Cancer Type	Clinical use	Cut-off concentration (ng/mL)	Year of discovery	Year of FDA-approval
CEA (carcinoembryonic Ag)	Colorectal	Monitoring	≈5	1965	1985
PSA (Prostate-specific Ag. PSA may be free or bound to other proteins)	Prostate	Monitoring	4–10	1979	1986
AFP(alfa-fetoprotein)	Testicular Ovarian Hepatocellular	Monitoring	10–200	1963	1992
fPSA(free PSA)	Prostate	Diagnosis	< 0.1 [PSA]	1995	1997
TG Thyroglobulin	Thyroid	Monitoring	≈1	1956	1997
CA-27–29	Breast	Monitoring	≈1–10*	1989	1997
CA-15-3	Breast	Monitoring	≈1–10*	1984	1997
CA-125	Ovarian	Monitoring	≈20	1981	1997
HER2	Breast	Monitoring HER2 positive tumor	≈15	1991	2000
CA-19-9	Pancreatic	Monitoring	≈1–10*	1979	2002
AFP-L3 (AFP isoform)	Hepatocellular	Diagnosis in patients with liver disease	≈30	1986	2005
FDP (Fibrin/fibrinogen degradation products)	Colorectal	Monitoring	≈600	1990	2008
HE4 (human epididymis protein)	Ovarian	Monitoring	≈0.3-0.4 ng/mL	2003	2008
Pro2PSA (PSA precursor isoform)	Prostate	Diagnosis	> f([fPSA],[tPSA])**	2001	2012

use [61]. The difficulty for finding biomarkers for early cancer detection reflects the lengthy and difficult path from biomarker discovery to clinical validation, and the lack of well-defined pipelines for this process.

In addition to the difficulties mentioned above, tumor-derived proteins in the circulation are at ultralow concentrations (< 1 pg/mL) during the early stages of tumor development. Current proteomic technologies are blind to these concentration levels buried by a complex mixture of more than 10,000 proteins, some of them at concentrations 11 orders of magnitude higher. Given that tumor-derived proteins are present at higher concentrations closer to their source, a logical route to discover protein biomarkers with current technologies is to detect cancer-associated proteins in the tumor tissue and proximal fluids. However, this approach does not guarantee the discovery of early cancer biomarkers that can be screened by simple blood-tests. Proteins found in the tumor can be unstable in circulation, associated with other proteins and subject to post-translational modifications. Moreover, the protein expression at the tissue level may not correlate with the relative concentration in blood. For instance, although *PSA* gene transcription is downregulated in prostate cancer, PSA protein levels in blood increase at early stages from 0.5–2 ng/mL to 4–10 ng/mL due to the destruction of the tissue architecture. In addition, cancer is a multistep disease that progresses through various phases resulting from the accumulation of genetic aberrations. The cancer biomarkers at early stages may not be the same as those at advanced stages, where most of the biopsies are taken.

Fig. 5 shows an estimation of the protein levels in plasma as a function of the tumor size, time after genesis of initial tumor cell and protein copies per cell. The calculation is based on recent reports aimed to quantify the proteome at the single cell level in mammalian and human cells [62–64]. These works estimate that the number of proteins per cell is of about $2–3 \times 10^9$, comprising protein copies from 10 up to 10^7, with a typical median value of 10^4. In most of the cells, 1000 most highly expressed proteins make up about 90% of the cell proteome. We assume a protein mass of 44 kDa that is the mean value in mammalian cells. We assume that the tumor growth follows a Gompertz function that has successfully described the growth of solid tumors [64,65]. In this model, we use a tumor-doubling time of 77 days and decaying rate of $2.5 \times 10^{-4}\,\mathrm{day}^{-1}$. These values are similar to those found in ovarian carcinoma. We assume that 10% of the proteins enter into the vasculature, similar to the secretion of CA-125 in ovarian carcinoma. For the sake of simplicity, we assume that the protein concentration in plasma reflects the concentration in cells. A more realistic model should account for the diverse mechanisms of protein elevation and elimination in plasma. However, this estimation is only intended to show the current technology limits and what can be expected by implementing recent ultrasensitive technologies.

In our model, the tumor reaches a size (diameter) of 20 mm 10 years after its genesis. These values are similar to those considered critical for initiation of metastasis [66,67]. We assess the capability of current techniques, MS and immunoassays, for detecting potential protein biomarkers shed by the tumor to the bloodstream. The limit of detection of these techniques is of about 1 ng/mL and 1 pg/mL, respectively. If the tumor markers are proteins highly expressed ($> 10^6$ copies), the tumor could be detected by MS with a size of about 12 mm, 9 years after its beginning, whereas immunoassays could detect the tumor with a size of 1 mm, five years after its beginning. In the case of intermediate expressed proteins, the tumor could be detected by MS and immunoassays with a size of about 50 mm and 5 mm, 12.5 and 7.5 years after its genesis, respectively. Low abundance proteins at the level of 100 copies per cell can only be detected by immunoassays, but more than 10 years after its genesis. So far, clinically approved protein biomarkers are found in the plasma at concentrations of ng/mL. With some notable exceptions, this protein repertoire is not useful for early cancer detection. The current technological limits prevent exploring the deepest part of the plasma proteome that arises from low abundant proteins in the tumor tissue. We here analyze the potential of nanomechanical systems for detecting ultralow concentration of proteins in plasma. For instance, as reviewed below, immunoassays based on nanomechanical systems are capable of detecting 50 ag/mL of protein biomarkers in plasma with very high repeatability [68,69]. This sensitivity implies that high abundance, intermediate abundance and low abundance proteins shed by the tumor could be detected in plasma when the tumor size is 50, 200 and 800 μm, respectively, at least five years before metastasis may occur. At this stage, most of the cancers can be efficiently treated and cured.

3. Nanomechanical systems for biomarker discovery

Current proteomics technologies cannot identify and detect proteins in plasma at concentrations below the pg/mL level. The deepest region of the plasma proteome is likely to contain proteins that can be used as future cancer biomarkers for early detection and population screening. It becomes clear that the development of ultrasensitive technologies for exploring the plasma proteome is demanding. Here, we describe recent advances on biodetection techniques based on nanomechanical systems

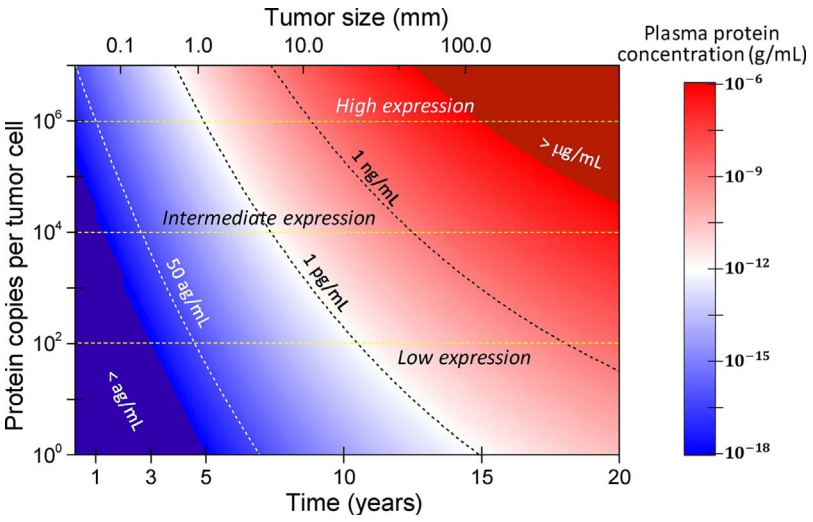

Fig. 5. Mathematical prediction of the plasma concentration of proteins shed by the tumor as a function of the time and protein abundance in the tumor cells. The relation between the time after the tumor inception and the tumor size is modeled by a Gompertz function. Parameters used in the model: plasma volume = 3490 mL, protein mass = 44 kDa, cell volume = 3000 μm^3, tumor volume to tumor cell volume = 4.5, tumor doubling time = 77 days, tumor decaying rate = 2.5×10^{-4} day^{-1}, ratio of protein biomarkers in plasma to protein biomarker in the tumor cells = 0.1.

that promise in the near future to complement the arsenal of proteomic-technologies.

3.1. Biosensors based on nanomechanical systems

Nanomechanical systems (NMSs) are devices that comprise a micro- or nanosized moving part whose natural oscillation frequency and position are sensitive to mechanical perturbations such as external forces and adsorption of substances. NMSs can be routinely fabricated by well-established processes in microelectronics, which guarantee mass production with highly reproducible features and low cost. NMSs shaped as microcantilevers are the most widespread devices by a conjunction of manifold reasons including simplicity, well-known theoretical models to interpret their response, low compliance and the capability for positioning the free end near the place of interest [70,71]. Microcantilevers are usually fabricated in silicon or silicon nitride with lengths from 100 to 500 µm, widths from 10 to 100 µm and thicknesses from 0.1-1 um (Fig. 6(a)). These devices can be miniaturized up to achieving suspended silicon nanowires, at least one million times lighter than standard microcantilevers (Fig. 6(b)) [72,73]. As shown below, reduction of the device dimensions is directly translated into an improvement of the detection limits. Large arrays with hundreds of devices can be easily fabricated for high throughput analysis and parallelization (Fig. 6(c)) [74]. NMSs shaped as doubly-clamped beams, trampolines and drums, to name a few, have been also used, although in less extent.

Biological detectors based on NMSs, referred to as nanomechanical biosensors, aim to measure the properties of biological components and their interactions in the mechanical domain [17–22]. Nanomechanical biosensors relies on measuring changes in the deformation (displacements) and vibrational properties of NMSs when adsorption of biomolecules or biomolecular interactions take place on the NMS surface [17]. The displacements of the nanomechanical system range from angstroms to hundreds of nanometers. These displacements can be measured by optical techniques such as the beam deflection method and interferometry that can provide detection limits well-below 0.01 nm $Hz^{-1/2}$. Electrical methods for the displacement readout can also be used although the sensitivity is reduced. Nanomechanical biosensors can be operated in the static or dynamic mode depending on whether the deformation or the vibration of the NMS is measured, respectively (Fig. 7). In the static mode, the displacement of the NMS, namely a microcantilever, is measured in real-time with a typical

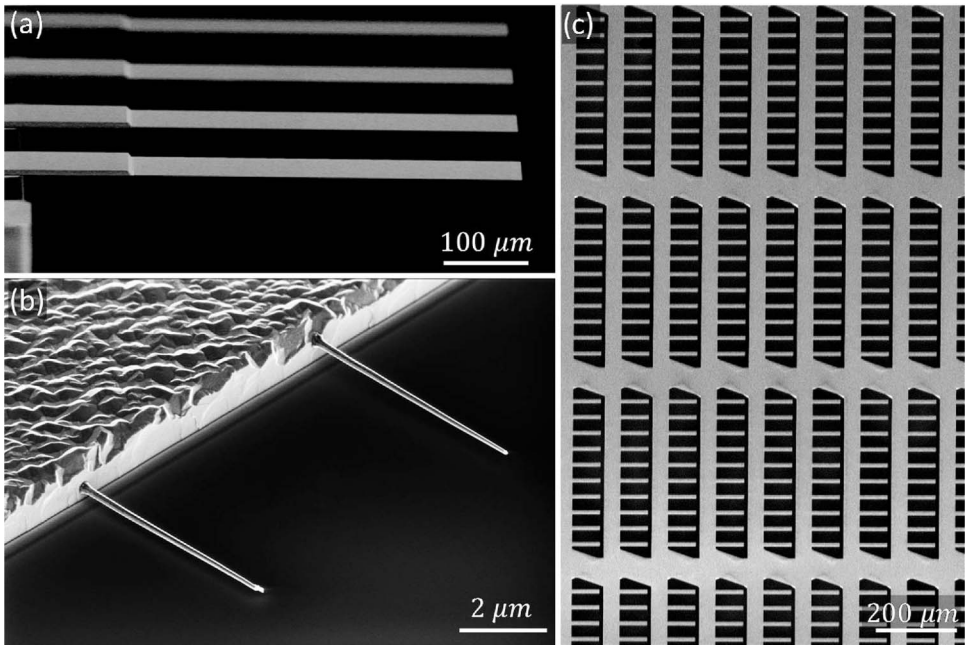

Fig. 6. Scanning electron microscopy images of several micro- nanocantilever sensors. (a) Silicon microcantilevers, 500 µm long, 100 µm wide and 1 µm thick. (b) Suspended silicon nanowires anchored to the wall of a microtrench. The length is of about 6 µm and the diameter of about 150 nm (c) Two dimensional array of silicon nitride cantilevers with a length of 60 µm, a width of 15 µm and thickness of 100 nm.

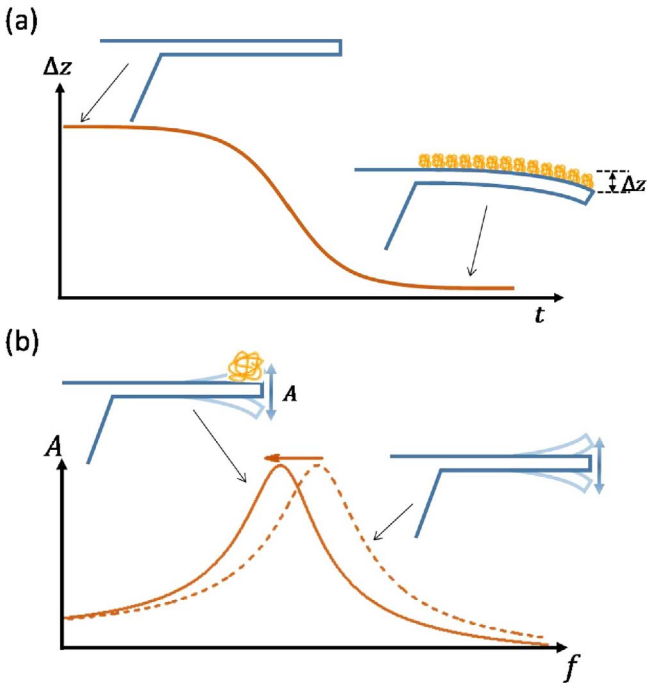

Fig. 7. Schematic of the most common operation modes of nanomechanical biosensors exemplified with a microcantilever. (a) In the static mode, one side of the cantilever is functionalized for immobilizing a monolayer of biomolecules. As the biomolecules form a monolayer, the cantilever bends due to the intermolecular forces parallel to the cantilever. (b) In the dynamic mode, the cantilever is driven by piezoelectric actuators, magnetic forces, and light-induced forces, to name a few. When a biomolecule lands on the cantilever, the resonance frequency shifts downwards due to the added mass.

averaging time of a second (Fig. 7(a)). The static mode is used for measuring biomolecular interactions in buffer solution on the surface of the NMS. A biological layer is confined to one of the faces of the NMS, called active face. The molecular interactions within the biomolecular components (electrostatic forces, hydrogen bonding, conformational entropic interactions, hydrophobic interactions) of the layer gives rise to surface stress [75–80]. The difference between surface stress of the active face and opposite face (passive face) induce the bending of the NMS.

In the dynamic mode, a NMS is driven to oscillate near or at the fundamental resonance frequency and variations of the amplitude or phase lag are monitored [17] (Fig. 7(b)). Phase-lock loops (PLLs) are usually implemented for keeping constant the phase lag and hence monitoring the resonance frequency in real-time. NMSs operated in the dynamic mode are usually referred to as nanomechanical resonators. Measuring in the time or frequency domains has the added advantage of not requiring calibration of the displacement measurements as it occurs in the static mode. Calibration of the static signal is a source of uncertainty. The dynamic mode has been traditionally used for mass sensing. The concept is that the resonance frequency of a NMS downshifts upon biological adsorption by an amount that is proportional to the ratio between the added mass and the NMS mass. Rapid advancements in micro- and nanofabrication technologies have allowed researchers to miniaturize the resonators to achieve increasingly smaller mass detection limits [17]. Mass records rapidly evolved from GDa (mass of *Escherichia coli*), achieved with a microcantilever in 2001 [81] to Da (10^{-24} g; the proton mass), achieved with a suspended carbon nanotube in 2012 [82]. The best detection limits have been obtained in high-vacuum conditions because the friction between the NMS and the environment is negligible, which implies better resolution in the resonance frequency measurement. Translation of these achievements to liquids, the natural environment for biology, remains elusive because of the very high energy loss in viscous environments [83,84]. When the thickness of the biomolecules is comparable to the thickness of the NMS, the stiffness of the biomolecules comes into play inducing an increase of the resonance frequency [71,85–87]. As shown below, this major complexity for interpreting the resonance frequency shifts can be converted into an opportunity for identification of biomolecules.

The choice among the static and dynamic measurement modes depend on the dominant mechanical signal in the biological assay, i.e., intermolecular forces, mass changes or stiffness variations. In some applications, the static and dynamic modes can be combined providing richer information on the biomolecular interactions. However, the optimal design of the NMS to achieve a highly sensitive static signal is different from the optimal design for obtaining a highly sensitive dynamic signal. Thus, a compromise in the design must be achieved. In addition, the simultaneous detection of the static and dynamic signal requires of specialized displacement measurement methods that avoid cross-talk between static and dynamic signals [88].

3.2. Methods for protein detection based on nanomechanical systems

In analogy to proteomics technologies, nanomechanical biodetection methods can be split into two broad categories: affinity-based methods and spectrometry methods. Affinity-based methods makes use of antibodies for detecting the target proteins, and hence requires prior knowledge on the expected protein content of the sample. Affinity based methods can be split into label-free assays and sandwich assays. Nanomechanical spectrometry identifies the target proteins by their physical properties. Depending on the signal processing algorithms, NMS properties and measurement method, nanomechanical spectrometry can be split into mass and mass and stiffness spectrometry. Nanomechanical spectrometry distinguishes itself from nanomechanical affinity assays in its potential for unbiased analysis of protein content.

3.2.1. Nanomechanical label-free affinity assays

This approach is based on the static mode method. The active face of a compliant microcantilever is coated with antibodies against the target proteins. The opposite passive face is blocked with antifouling molecules to minimize nonspecific adsorption. The surface stress induced by the antibody-antigen binding induces the microcantilever displacement that is detected by either optical or electrical methods. The measurements can be performed in real-time [76,77,89,90] or, alternatively, before and after incubation of the microcantilever in the test sample to perform end-point detection of the target proteins [12]. Detection in real time provides information about the kinetics of the biomolecular interactions, while end-point detection is usually deemed more practical for applications where the need is limited to detecting the presence of a protein and its concentration. Endpoint detection is also more suitable for multiplexing. Label-free nanomechanical affinity assays attracted worldwide attention at the beginning of new millennium due to its capability for detection of sub-ng/mL concentrations of biomolecules with no need of labels for quantification. Despite worldwide huge efforts, the promise of applications of this technology in the biomedical field has been frustrated because of its low reproducibility. With some notable exceptions, works lack of both thorough statistical analysis and appropriate validation methods that are widely use in standard immunoassays to assess robustness, precision, limit of detection, limit of quantification, inter- and intra-assay variability, selectivity, probability of false positives and false negatives, etc [12]. Related to these problems is the mechanism of generation of the nanomechanical signal. Intermolecular forces due to molecular recognition are weak, and the induced surface stress signal can be in most of the cases buried by nonspecific signals such a nonspecific adsorption, small variations in the environmental conditions (temperature, pH, etc) and instrumental artefacts (thermal drift). More importantly, the generation of detectable amounts of cantilever deflection requires highly crowded surfaces in order to maximize the repulsive physical steric

interactions, ion osmotic pressure and hydration forces [17,79]. These conditions are difficult to obtain in a reliable way. The label-free capability of affinity nanomechanical biosensors is remarkable and can be harnessed for studying biomolecular interactions without the perturbative effect of tags. However, this class of nanomechanical biosensor has limited capability for human proteomics.

3.2.2. Nanomechanical sandwich immunoassays

This method is based on the dynamic mode described above. The surface of the NMS is functionalized with capture antibodies. The functionalized NMS is first incubated with the sample to allow binding of the targeted protein biomarker to the capture antibodies immobilized on the NMS surface. Second, after stringent rising of the NMS to remove nonspecific adsorption, the NMS is incubated with a solution of nanoparticles, usually gold nanoparticles, functionalized with the detection-antibodies that recognize a free region of the captured biomarker. The NMS is rinsed and dried. The nanoparticle acts as a mass label. The resonance frequency of the NMS is measured before and after the immunoassay in air or vacuum. The mass of the nanoparticles induces a significant downshift of the resonance frequency. This method allows counting the number of nanoparticles on the surface of the NMS. The use of two antibodies together the advantageous features of the dynamic mode, largely enhance the selectivity, reproducibility and the limit of detection. In 2009, Craighead and co-workers successfully used this method with trampoline-like NMSs for detecting PSA in serum, achieving a detection limit of 0.05 pg/mL [91]. In 2014, Kosaka and collaborators used arrays of microcantilevers and 100-nm diameter gold nanoparticles for detection of the cancer biomarkers PSA and CEA in serum [69] (Fig. 8). A detection limit of 10^{-4} pg/ml was achieved with both biomarkers, which is at least fiver orders of magnitude lower than that achieved in routine clinical practice. The implications of this ultrasensitivity in early cancer detection are discussed above (Fig. 5 and related text). Kosaka's method offers an added advantage: the microcantilevers can be used as optical cavities that boost the light scattered by the gold nanoparticles (a process known as cavity-enhanced plasmonic transduction). Simple inspection of the microcantilevers by a simple optical microscope in the dark-field mode allows distinguishing the presence of ultralow concentration of biomarkers in the sample. Combining nanomechanical and nanooptical quantification in a single device provides major robustness (two eyes are better than one) and higher reliability, achieving rates of false positives and false negatives in controlled samples of $\sim 10^{-3}$. Refinement of the technology has allowed detection of 10^{-5} pg/mL of the HIV-1 capsid antigen p24 in human serum [68]. The technique was adapted to the common medical diagnostic format by carrying the immunoassay steps in a 96-well microtiter plate format, which allows detection of multiple samples in the same plate. The technology is also suitable for high degree of parallelization and to detect multiple biomarkers by using dense microarrays of microcantilevers and dedicated microfluidics [74].

3.2.3. Nanomechanical mass spectrometry

In 2012, nanomechanical resonators were used for the first time for real-time mass spectrometry of proteins [92,93]. Similarly to MS, the species were introduced by either electrospray ionization or matrix-assisted laser desorption ionization (MALDI). Subsequently, the ionized species are guided by ion optics to the nanomechanical resonator in high vacuum ($< 10^{-5}$ Torr). As each analyte adsorbs on the mechanical resonator, an abrupt resonance frequency downshift is observed that is proportional to the ratio of the analyte mass to the device mass with a proportionality constant that depends on the adsorption position. The mass and adsorption position were decoupled by simultaneous measurement of the first two vibration modes of the resonator, a doubly clamped nanobeam. Nanomechanical mass spectrometry has been successfully applied for '*weighting*' BSA (66 kDa), β-amylase (200 kDA) and immunoglobulin M (960 kDa) in purified solutions. The mass resolution was of the order of 10 KDa, approximately. This resolution is still very far from that achieved in conventional MS. Nanomechanical mass spectrometry can readily achieve higher mass resolution ($\sim$10 Da) by miniaturizing the employed nanomechanical resonator. However, the capture efficiency, that is inversely proportional to the resonator area, rapidly degrades with miniaturization. It is expected that this limitation will be overcome in the next years. Advances in micro- nanofabrication technologies are mature enough to provide highly dense two-dimensional arrays of nanomechanical resonators ($>$ 1000 devices) operating in parallel in order to increase the capture efficiency without losing mass sensitivity [94,95].

3.2.4. Nanomechanical mass and stiffness spectrometry

Theoretical models had predicted that the stiffness of the analyte influences on the resonance frequency variations recorded in nanomechanical spectrometers [17,85,87] (Fig. 9(a)). This effect is particularly relevant when using ultrathin nanomechanical systems ($<$ 100 nm) or when the analyte size is comparable to the beam thickness. In 2016, a custom-built nanomechanical spectrometer proved the capability for resolving the mass and stiffness of analytes landing on the surface of a microcantilever [96]. Electrospray ionization was used to generate mostly desolvated charged species at ambient pressure, and subsequently the charged species were guided to the

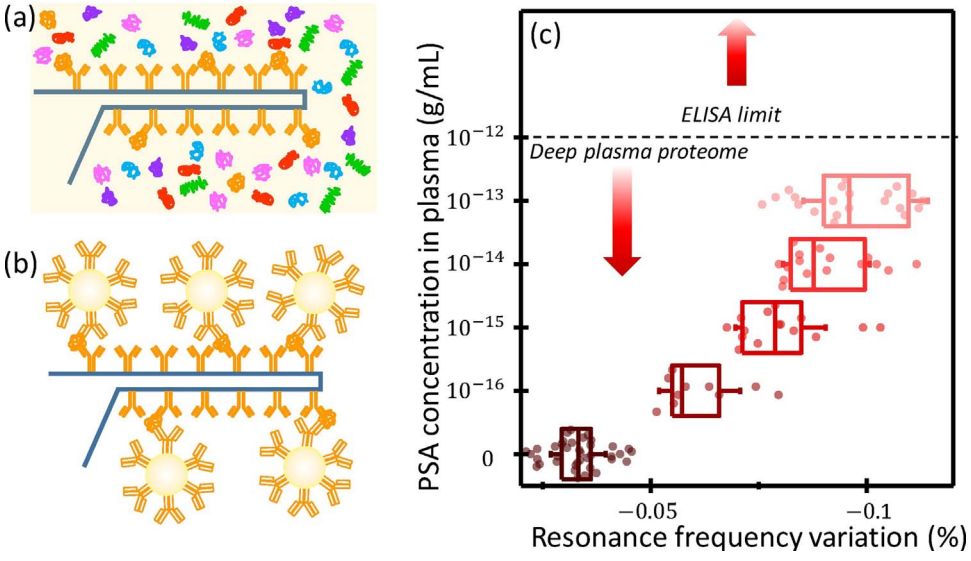

Fig. 8. Left: schematic representation of a nanomechanical sandwich immunoassay. (a) The microcantilever functionalized with capture antibodies is immersed in the human plasma sample to allow specific binding of the sought protein. (b) The protein biomarker captured on the cantilever is specifically linked to gold nanoparticles that carry detection antibodies. The diameter of the gold nanoparticles can range from 50 to 100 nm. (c) Example of PSA detection in human plasma (unpublished results of the authors). The resonance frequency of the microcantilevers downshifts by an amount that is proportional to the ratio between the mass added by the nanoparticles and the microcantilever mass. The results illustrate how this technology can explore the deepest region of the plasma proteome.

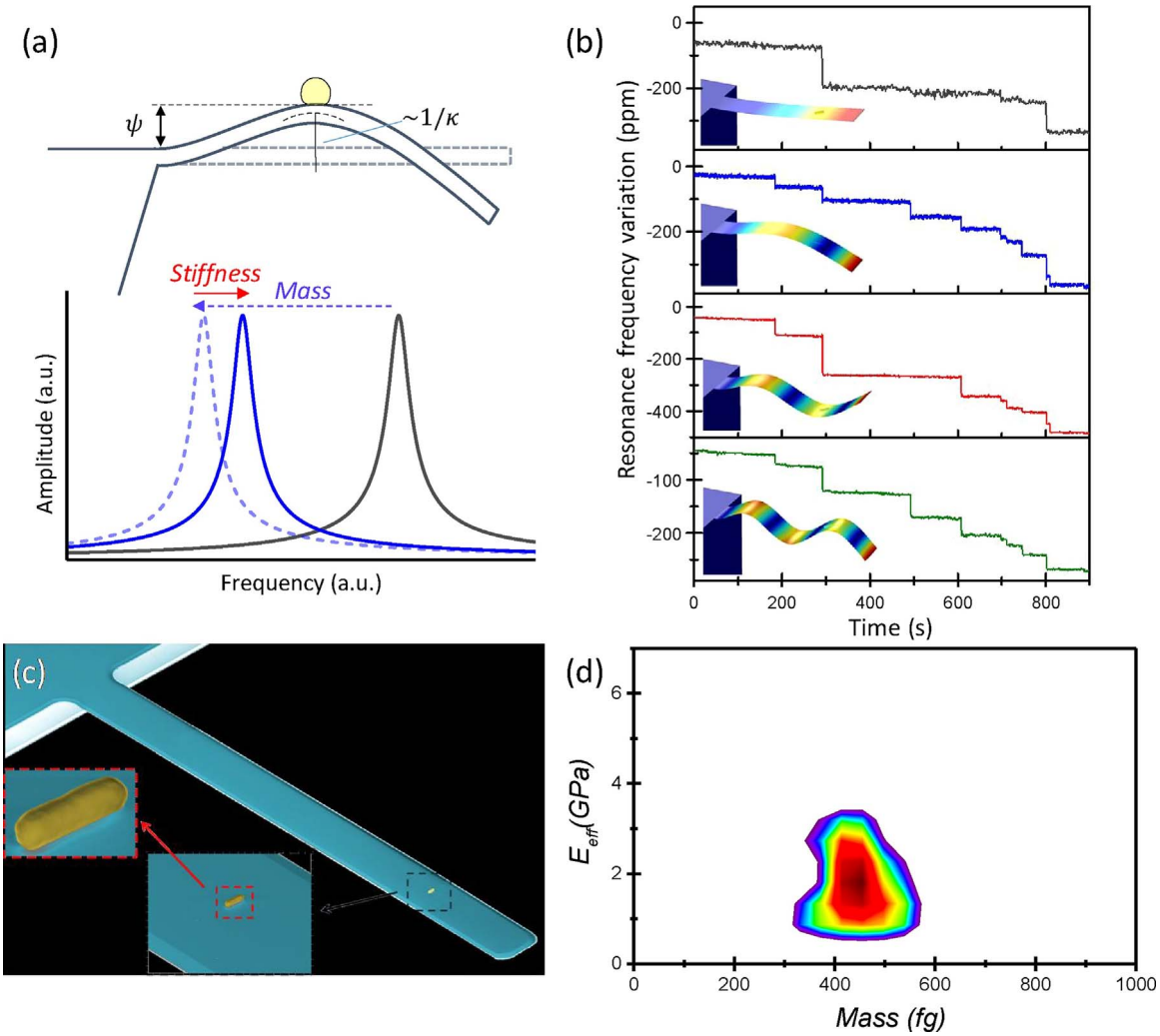

Fig. 9. (a) Schematic depiction of an adsorbate on a cantilever vibrating at the resonance frequency of the second vibration mode. The stiffness of the adsorbate induces a positive shift of the resonance frequency. Although the overall shift of the resonance frequency is negative, accurate quantification of the mass of the adsorbate requires accounting for the stiffness effect. (b) Real-time record of the fractional shifts of the resonance frequencies of the first four vibration modes of the microcantilever during bacteria adsorption. The shapes of the vibration modes of the cantilever are shown in the insets. The time-correlated frequency jumps in the graphs correspond to adsorption events of single bacterial cells. (c) SEM image of an *E. coli* cell on the microcantilever. The insets show closer views of the bacterium. (d) Nanomechanical fingerprint of the E. coli cells corresponding to 189 adsorptions. E_{eff} is the effective stiffness of *E. coli* cells.

microcantilever at low vacuum by pressure gradient through three chambers with decreasing pressure. The proof of concept was demonstrated with gold nanoparticles and bacteria *E. coli*. Fig. 9(b) shows the abrupt jumps in the vibrational properties of the microcantilever as individual bacterial cells land on the vibrating microcantilever. The technique allows measurement in low vacuum, which makes that the biological analyte arrive intact and near native conformation (Fig. 9(c)). Numerical algorithms allow translation of the variations of the resonance frequencies into the mass and stiffness of each bacteria (Fig. 9(d)).

Nanomechanical spectrometry is still in its infancy and far from implementation in proteomics. However, we foresee rapid developments in the next years that can place this technology close to the needs in proteomics. The mass resolution is still very far from that obtained in MS, although it can be readily improved to 100 Da with existing devices. Mass spectrometry measures the mass-to-charge ratio of molecular species from 100 Da to 100 kDa with extremely high accuracy. At this level, mass spectrometry is unbeatable. However, its performance largely degrades on the measurement of heavier species, such as intact proteins and protein complexes. The application of MS in proteomics requires of the fragmentation of the proteins into small peptides. The MS and MS/MS patterns become difficult to interpret and in addition, the information on the biological processes such as protein–protein interactions is lost. Experiments of nanomechanical spectrometry with silicon nanowires (Fig. 6(b)) and inorganic adsorbates have demonstrated the capability to simultaneously achieve high resolution in mass and stiffness. Translation of these results to proteins imply identifying proteins with a mass resolution of 1 kDa and detecting variation in the Young's modulus of the protein of 0.1% [72,73]. It is well-known that subtle protein changes such as single mutations that may be undetectable by mass are manifested as biologically relevant mechanical changes. Nanomechanical spectrometry could be applied for detecting these modifications. Another virtue of nanomechanical spectrometry is that the nanomechanical signature is insensitive to the charge of the analyte, simplifying the analysis of the data [97]. The enormous number of charge states obtained in MS produces complex spectra with overlapping peaks that are often difficult to interpret. Only time will tell if advancements in nanomechanical spectrometry will be able to tackle the acute problems in proteomics for the discovery of useful protein biomarkers for early cancer detection. Meanwhile, a European initiative involving several laboratories with multidisciplinary expertise has been launched in order to achieve a nanomechanical spectrometer able to identify harmful viruses by mass and stiffness from blood samples in clinical setting (http://viruscanproject.eu/). If this project succeeds, we

will soon witness the first generation of nanomechanical spectrometers for human proteomics.

4. Conclusions

Early cancer detection will be achieved once that we can identify the protein biomarkers shed by the tumor to the bloodstream since its inception. Proteomic technologies, mainly mass spectrometry and multiplexed immunoassays, have rapidly developed during recent years with improved limits of detection and multiplexing capability. They can reliably explore the plasma proteome up to the ng/mL level, and in some cases to the pg/mL level. Still, thousands of unidentified proteins are expected to be discovered in the human plasma with major refinement of these proteomic technologies. However, access to the deepest region of the proteome will require of new ultrasensitive technologies capable of quantitating proteins in the plasma at concentrations several orders of magnitude lower than 1 pg/mL. This region of the plasma proteome is likely to contain specific protein biomarkers for early cancer detection. Here we propose the development of biological detectors based on nanomechanical systems for their incorporation into the technological arsenal of human proteomics. In particular, we point out to two recent developments, nanomechanical sandwich immunoassays and nanomechanical spectrometry. The first technique enables reproducible immunodetection of proteins at concentrations well below the pg/mL level, with a limit of detection on the verge of 10 ag/mL. This technology can potentially detect low abundance tumor-associated proteins at the very early stages of the tumor. Its level of development enables its rapid integration into the protein biomarker discovery pipeline. The second technique enables the identification of individual proteins by two physical coordinates, the mass and stiffness of the intact protein. Although still far from implementation in proteomics, it can easily benefit from the already MS advanced technologies for protein separation, nanoelectrospray ionization and efficient transport of protein ions in vacuum to the detector. We believe that in the near future nanomechanical protein detectors will play a fundamental role for deciphering the deepest region of the human plasma proteome. Let us cross the fingers for witnessing the discovery of specific cancer biomarkers that can save millions of lives every year by using a simple blood test.

Conflicts of interest

The authors declared no conflict of interest.

Acknowledgments

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